IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Mikio SUZUKI, et al.

Serial No.: 10/505,153 Art Unit: 1635

Filed: March 14, 2005 Examiner: Jene Zara

For: POLYNUCLEOTIDE FOR TARGET GENE

DECLARATION

Honorable Commissioner of Patents and Trademarks

Washington, D. C. 20231

SIR:

I, Hiroshi MOMOTA, declare that:

1) I am one of the inventors of the above-identified application, and am familiar with the subject matter of said application as well as the disclosures in the cited reference.

2) The experiments given below were carried out under my general direction and supervision.

ADDITIONAL EXPERIMENT

1. Purpose

Supporting activity of stem-loop-type siRNA expression vectors on gene expression was demonstrated below. Specific interference of gene expression was observed as the chemically synthesized small interfering RNA (siRNA) for a target gene was introduced into mammalian cells. Vectors constructed to produce siRNAs for specific genes were considered to show the same effect as chemically synthesized siRNAs in mammalian cells. We constructed the stem-loop-type siRNA expression vectors for firefly luciferase gene (F-Luc) and evaluated them on the suppression of F-Luc expression.

2. Experimental procedure

Synthesis of stem-loop units for luciferase gene: DNA oligonucleotides were synthesized. Synthesized DNA sequences that comprised components (I), (II), (III), and additional nucleotides for connecting to vectors were,
U6H1-sas; 5′-CTCGAGAAAAA - GCATAAGGCTATGAAGAGATACGCC(I) - CTTAAG(II) - GGCGTATCTCTTCATAGCCTTATGC(III) - TTTTTGGTCTAGA-3′, and U6-ass; 5′- TTCGAAACACC - GGCGTATCTCTTCATAGCCTTATGC(III) - CTTAAG(II) - GCATAAGGCTATGAAGAGATACGCC(I) - TTTTTGGTCTAGA -3′.

Construction of stem-loop-type siRNA expression vectors: The synthesized stem-loop units were digested with XhoI (Takara) and XbaI (Takara), recovered by MiniElute column (QIAGEN), and then inserted in stem-loop type siRNA vector digested with XhoI and XbaI. The constructed plasmid were transfected in E. coli, then selected by their ampicillin resistance.

Preparation of cells: HeLa S3 cells were pre-incubated in DMEM medium (Sigma) supplemented with 10% FBS (JRH). One day before transfection, cells were recovered and plated at 10^5 cells per well in a 24-well plate (Falcon).

Preparation of transfection complexes: For each transfection sample, 0.09μg of pGL3-control (expression vector for F-Luc, Promega), 0.01μg of pRL/TK (expression vector for Renilla-Luc as an internal standard, Promega), and 0.9μg of stem-loop-type siRNA vector were suspended in 50μl of OPTI-MEM I (Invitrogen). In another tube, 3μl of Lipofectamine 2000 (Invitrogen) was suspended in 50μl of OPTI-MEM I. The plasmid mixture and Lipofectamine 2000 solution were then mixed and stand for 20min at room temperature.

Transfection: 100μl of transfection complexes were added to each well of the 24-well plate. The cells were incubated at 37°C in a CO_2 incubator for 24 hours.

Measurement of luciferase activities: Cells were once washed with PBS(-) and then treated with passive lysis buffer (Promega). Luciferase activities were measured with Dual-Luciferase Reporter Assay System (Promega). The activity of F-Luc was normalized by Renilla luciferase activity (R-Luc) to exclude the effect of transfection efficiency.

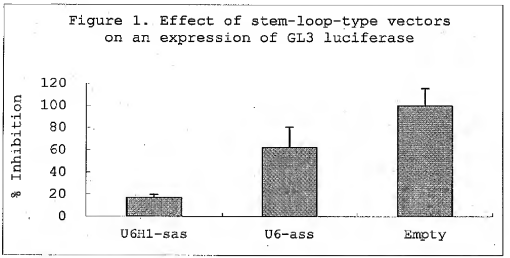
3. Results

Specific knockdown for luciferase activity was determined as follows. Stem-loop type siRNA vectors were cotransfected in HeLa S3 cells with pGL3-control and pRK/TK. After incubation for 24 hours, luciferase activities were analyzed. Table 1 shows the results of luciferase assay. The effects of stem-loop-type siRNA vectors are summarized in Figure 1. Vectors, U6H1-sas and U6-ass show an inhibitory effect on luciferase activity compared with an empty vector. We concluded that the stem-loop-type siRNA vectors were useful tool for specific gene knockdown.

Table 1

	F-Luc	R-Luc	F-Luc /R-Luc	% Inhibition	Stdev
U6H1-sas	2361	33624	0.070	17.1	2.6
U6-ass	2106	8448	0.256	62.4	18.3
Empty	6128	14990	0.410	100	15.7

n=3



I, the undersigned, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Jan. 09. 2008 Hiroshi Momota

Hiroshi Momota